ISSN 0704-3716

Canadian Translation of Fisheries and Aquatic Sciences

No. 5519

Method of measuring the diarrhetic shellfish toxicity of scallops and comparative examination of the virulence values

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Original title: Hotategai Gerisei Kaidoku Sokutei-ho oyobi Dokuryokuchi no Hikaku Kento.

In: Aomori-ken Eiseikenkyusho-ho/Bull. Aomori Prefect. Health Res. Cent. 26: 45-52, 1989.

Original language: Japanese

Available from:
Canada Institute for Scientific and Technical Information
National Research Council
Ottawa, Ontario, Canada K1A 0S2

1990

22 typescript pages

91-01292/

Secretary of State / Secrétariat d'État

MULTILINGUAL SERVICES DIVISION – DIVISION DES SERVICES MULTILINGUES

TRANSLATION BUREAU BUREAU DES TRADUCTIONS

LIBRARY IDENTIFICATION – FICHE SIGNALÉTIQUE

Translated from - Traduction de	Into - En
Japanese	English

Author - Auteur

Akiko FURUKAWA, Mami NOMURA, Junko MURAKAMI and Hideichi KOBAYASHI

Title in English or French - Titre anglais ou français

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Title in foreign language (Transliterate foreign characters)
Titre en langue étrangère (Transcrire en caractères romains)

Hotategai Gerisei Kaidoku Sokutei-ho oyobi Dokuryokuchi no Hikaku Kento

Reference in foreign language (Name of book or publication) in full, transliterate foreign characters.
Référence en langue étrangère (Nom du livre ou publication), au complet, transcrire en caractères romains.

Aomori-ken Eiseikenkyusho-ho

Reference in English or French - Référence en anglais ou français

Bulletin of the Aomori Prefectural Health Research Center

Publisher - Editeur	DATE OF PUBLICATION / DATE DE PUBLICATION			Page Numbers in original / Numéros des pages dans l'original
	Year / Année	Volume	Issue No. / Numéro	45 - 52
Place of Publication / Lieu de publication	1989		26	Number of typed pages / Nombre de pages dactylographiées: 20

Requesting Department / Ministère-Client: DFO

Translation Bureau No. / Notre dossier nº: 3850522

Branch or Division / Direction ou Division: Biological Station, St. Andrews, New Brunswick E0G 2X0

Translator (Initials) / Traducteur (Initiales): PS

Person requesting / Demandé par: Dr. V. Zitko

Your Number / Votre dossier nº:

Date of Request / Date de la demande: October 19, 1990

DEC 11 1990
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Canada

SEC 5-111 (84-10)

DEC 11 1990

Method of measuring the Diarrhetic Shellfish Toxicity of Scallops and Comparative Examination of the Virulence Values

by Akiko FURUKAWA, Mami NOMURA, Junko MURAKAMI and Hideichi KOBAYASHI

in Aomoriken Eiseikenkyusho-ho (Bulletin of the Aomori Prefectural Health Research Center) , **26**, 45-52, 1989

Foreword

To measure the diarrhetic shellfish poison of scallops, one uses presently the official method using mice as experimental animals[1]. However, there are problems with regard to the keeping of mice, with regard to detection sensitivity, precision and such. Moreover, it is impossible to measure the shellfish poison components separately [2) 3)].

There are reports of instrumental analysis methods [3-8)] and of epidemiological methods[9)] that compensate the defects of the above methods.

The authors examined previously a method of measuring diarrhetic shellfish poisons which is based on the high-performance liquid chromatographic (denoted hereafter as HPLC) method, and they published a report about the same [10)].

In order to establish a rapid and highly sensitive method of measuring the virulence of diarrhetic shellfish poison, we made comparisons of the virulence values of the components causing diarrhea based on the HPLC method, on the enzyme epidemiological method, as well as of the virulence values based on the official method. We also examined the effect of the free fatty acids at low virulence time and obtained some insights which we report hereunder.

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Examination Method

1. The samples

We used the midgut glands of cultured scallops gathered at a perpendicular depth of 20 m at sites off Nonai and Nobeji in the Mutsu Bay sea area.

2. Points surveyed

Figure 1. shows the points of survey.

3. Duration of the survey

March 1988 - December 1988

4. Samples

Okaidic acid (OA) and Dinophysistoxin-1 (DTX_1) standard solutions : We dissolved in chloroform standard preparations donated by Professor Yasumoto of the Tohoku University and adjusted to the 0.1 µg/mL concentration.

9-Anthryldiazomethane (ADAM) : We used a preparation from the Funakoshi Pharmaceuticals Co. in a 0.1 % methanol solution.

Sep-pak silica cartridge : We used a Sep-pak silica cartridge from the Waters Co. after allowing for impregnation with a chloroform-hexane (1:1) solution.

DSP-Check (handy diarrhetic shellfish poison measurement kit) : From the Ube Kosan Co.

5. Equipment

High-speed liquid chromatograph : Model 638 of Hitachi

Detector : Fluorescence detector (Model 650-10 spectrofluorescence photometer from Hitachi)

Gas chromatograph : Shimadzu GC-9 A

Detector : FID

UBE Handy Reader : Ube Kosan Co.

Biotron : BT 10-35 type made in Switzerland

Rotary evaporator : Yamato RE-51

Homogenizer : Nippon Seiki Sakusho Co. (precision instruments)

6. Measurement conditions

(1) HPLC

Same as in previous report [10]

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(2) GC

Column : Diasolid ZF, 80-100 mesh, 3mm x 2m (made of glass)

Column temperature : 190 °C

Detector temperature : 250 °C

N_2 flow rate : 50 mL/min

7. Method

In order to inhibit the enzymatic activity of lipase and such, we warmed the midgut glands used for measurements with an electronic range, after which we homogenized and used as sample. p. 46

(1) The official method

Following the diarrhetic shellfish poison examination method (Ministry of Health and Welfare, Environmental Health Bureau, Milk and Meat Health Division), we performed the acetone extraction of the midgut glands, and after allowing to dry, performed the ether-water partition. After the ether layer had dried to hardness, we used the obtained product for the mouse tests.

(2) The HPLC method

We performed measurements by the method explained in our former report [10], and according to the report by Yasumoto [6] we converted to virulence values by taking the 4 μg reading as MU.

(3) The enzyme immunity method (DSP-Check method) [9]

a. The OA, DTX_1 virulence value

The measurement method is shown in Fig. 2.

We extracted the midgut glands with a 90 % methanol solution, measured the OA concentration by the antigen-antibody reaction, using a sample solution obtained by diluting twice the supernatant after centrifugation, and converted to virulence value.

p. 47

However, as the monoclonal antibody used in the present method has the same cross reactivity with regard to OA, DTX_1, all of the OA and DTX_1 in the sample solution is measured in terms of OA.

b. DTX_1 + DTX_3 virulence values

The measurement method is shown in Fig. 3.

We proceeded as with the HPLC method. This means that we performed alkaline degradation of the ether solution treated according to the official method, using a 90 % methanol solution containing 0.5N sodium hydroxide, and after converting all DTX_1 and DTX_3 to DTX_1, we neutralized with acetic acid and performed the ether extraction. After setting the volume of the ether layer, we took half of the same, removed the ether, measured thereafter as DTX_1 + DTX_3 while proceeding as with OA, DTX_1, and then converted to virulence values.

(4) Free fatty acid concentration [11]

The measurement method is shown in Fig. 4.

We treated the midgut glands according to the official method, and after setting the volume of the ether layer, collected a portion of the same, methylated with diazomethane after which we measured by the GC method. To determine the volumes, we used heptadecanoic acid methyl esters (C_{17}) as standard substance, and performed the measurements on the basis of the total volume of the peak areas of 10 types of fatty acid (C 14:0, C 16:0, C 16:1, C 18:0, C 18:1, C 18:2, C 18:3, C 20:4, C 20:5, C 22:5, C 22:6).

Results and discussion

We show in Tables 1, 2 and in Figures 5-8 the various virulence values obtained by the official method, the HPLC method and the DSP-Check methods at the Nonai and Nobeji sites. As we did not have enough samples for the DSP-Check method, some measurement were not possible.

1. OA, DTX $_1$ virulence values at the Nonai site

When we observe the changes in virulence based on the official method, we see that in March, April the changes were less than 0.3 MU/g, that there was a sharp increase from June (0.6 MU/g), and a gradual decrease thereafter.

When comparing the virulence values based on the DSP-Check method and the HPLC method, we detected with the DSP-Check method a virulence of less than 0.2 MU/g during the low-toxicity period of March, April and of October-December. However, no DTX $_1$ was detected by the HPLC method.

In the high-toxicity period going from June to August, the DTX $_1$ virulence based on the HPLC method had a value about 10 % that of the official method.

At no time, was OA detected by the HPLC method.

2. The DTX$_1$ + DTX$_3$ virulence values at the Nonai site

When we compare the virulence values based on the HPLC and DSP-Check methods, we see that in spite of some differences during the high-toxicity period of June-July, the overall patterns are similar.

Next, from May to September, the virulence values based on the DSP-Check method were about 60 - 100 % those based on the official method values, and the values were higher than in the winter.

Furthermore, if we consider the proportion of DTX $_3$ vs. DTX $_1$ + DTX $_3$, we note 0- 40% for March, April, and about 60-100 % for May and later, and we see that the proportion of DTX $_3$ was large from the summer through the winter.

The differences from the virulences based on the official method are estimated to be due to the toxic components other than the diarrhea-inducing components.

3. The OA, DTX $_1$ virulence values at the Nobeji site p.48

If we consider the changes in virulence based on the official method, we note changes with low virulences of 0.4 MU/g or less in the spring, but see also that the normal values (0.05 MU/g for the edible parts) were exceeded on March 22 (denoted as 3/22; same hereafter), which is about 40 days sooner than at the Nonai site [12]. Definite toxification sets in from June. After a peak value of 8.0 MU/g exhibited on 7/4, there is a temporary drop followed by a new increase to 6.0 MU/g on 8/8. The virulence drops thereafter but on 12/5, one detected a high virulence of 2.0 MU/g absent in normal years.

The two virulence peaks noted in the summer at the Nobeji site were observed to continue here for several years [13,14].

If we compare the virulence values based on the DSP-Check and the HPLC methods, we noted that with the DSP-Check method, the detected virulence during the low-toxicity period of March-May is 0.2 MU/g or lower like at the Nobeji site, and that no DTX $_1$ was detected with the HPLC method.

Next, during the high-toxicity period going from June to August, the DTX$_1$ virulence values based on the HPLC method were about 6-20 % of those based on the official method.

Meanwhile, at no time was OA detected by the HPLC method.

4. The DTX$_1$ + DTX $_3$ virulence values at the Nobeji site

The virulences based on the HPLC and the DSP-Check methods showed fairly similar values.

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Moreover the virulence values based on the official and the DSP-Check methods reveal roughly similar patterns, but with the DSP-Check method on the 8/8, the values were as low as about 40 % of those based on the official method.

In the survey made by the Fisheries Department of the Aomori Prefecture, it is reported that the appearance of *D. fortii* believed to be the plankton causative of DTX_1 and the changes in virulence correspond practically 15).

Therefore, these differences in toxicity cannot be explained solely through the numbers of plankton, and it is necessary to examine the unit toxin volumes, the PTX components and such per 1 cell of *D. fortii*.

If then, we consider the proportion of DTX_3 vs. $DTX_1 + DTX_3$, we see that it was 0 - 30 % in March-May, and about 60-90 % from June and thereafter, and that like at the Nonai site, the proportion of DTX_3 was larger from the summer through the winter.

The reason is that DTX_1 only is present in the plankton, and it is estimated to be converted to DTX_3 inside the midgut glands of the scallops 3), which suggests that the metabolism progresses more actively in the summer and later than it does in the spring.

p. 49

5. Correlation between the virulence values of the DSP-Check and of the HPLC methods

Fig. 9 shows the correlation between the virulence values of the DSP-Check and the HPLC methods with regard to 34 $DTX_1 = DTX_3$ samples for both the Nonai and the Nobeji sites. A fairly good correlation is noted, the correlation coefficient being 0.989.

6. The free fatty acid concentration

Neither OA or DTX_1 was detected in the spring at the Nonai site by the HPLC method, but the official method revealed a low virulence. So, we measured by the GC method the concentration of free fatty acids (denoted hereafter as FFA) judged to exhibit mouse toxicity. The results are shown in Table 3 and Graph 10.

The concentration ranges were 0.11-6.59 mg/g at the Nonai site, and 0.58-5.78 at the Nobeji site. The two sites showed similar patterns. The concentrations were also lower in the summer and later than in the spring.

The FFA concentration having an effect upon mice is estimated to be 12 mg/g (FFA volume of 40 mg when administering 1 mL or more to mice) 11). It thus seems that at this concentration, the effect is virtually nil.

Conclusion p. 51

In order to establish a rapid and highly-sensitive method of measuring the virulence of diarrhetic shellfish poison, we compared the virulence values of the diarrhea-causing components based on the HPLC and the DSP-Check methods on the one hand to the virulence values based on the official method on the other.

1. With regard to $DTX_1 + DTX_3$ there is a fairly good correspondence between the virulence values based on the DSP-Check and the HPLC methods. The correlation coefficient was 0.989.

2. As for DTX_1, we detected a virulence of no more than 0.2 MU/g with the DSP-Check method during the low-virulence period of the spring. Nothing was detected with the HPLC method.

No OA was detected by the HPLC method.

3. When comparing the $DTX_1 + DTX_3$ virulence values base on the HPLC and the DSP-Check methods to the virulence values based on the official method, the observed patterns were on the whole similar. The differences from the virulence values based on the official method are estimated to originate from toxic components other than those causing diarrhea.

4. When comparing the proportions of DTX_1 and DTX_3, we noted that at both sites DTX_1 was high in the spring and that DTX_3 was higher from the summer and later.

5. In the present survey, the FFA concentrations were 0.11-6.59 mg/g at Nonai, and 0.58-5.78 mg/g at Nobeji. At these concentrations, there seems to be virtually no effect of the FFA upon the virulence values.

6. The present results suggest that when monitoring the virulence of the sole components causing diarrhea, it should be possible to use the DSP-Check method as the procedure is simple, rapid as well as highly sensitive. It seems that in order to make it also practical, one should gather compared data regarding the official method as well as regarding the HPLC and DSP-Check methods.

図1　調査定点

Figure 1　The sites of survey

a. Sea of Japan, b. Mutsu Bay, c. Nonai site, d. Nobe site, e. Pacific Ocean, f. Nobeji, g. Aomori City

Fig 2 Method of determining the OA, DTX_1 concentrations

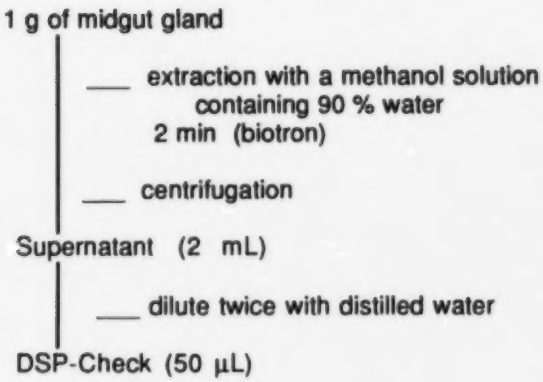
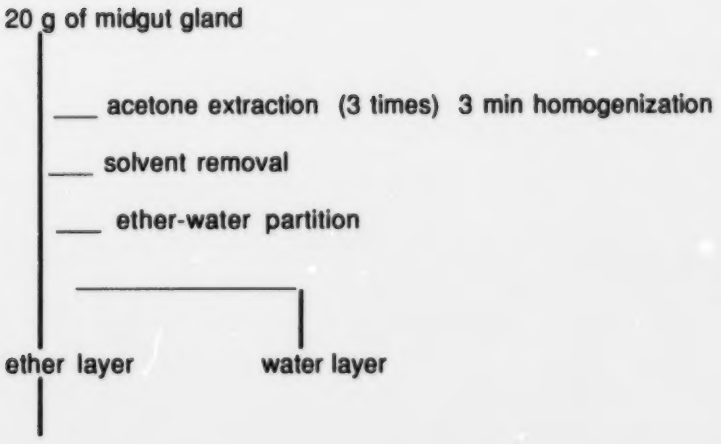


Fig. 3 Method of measuring the (DTX_1 + DTX_3) concentrations



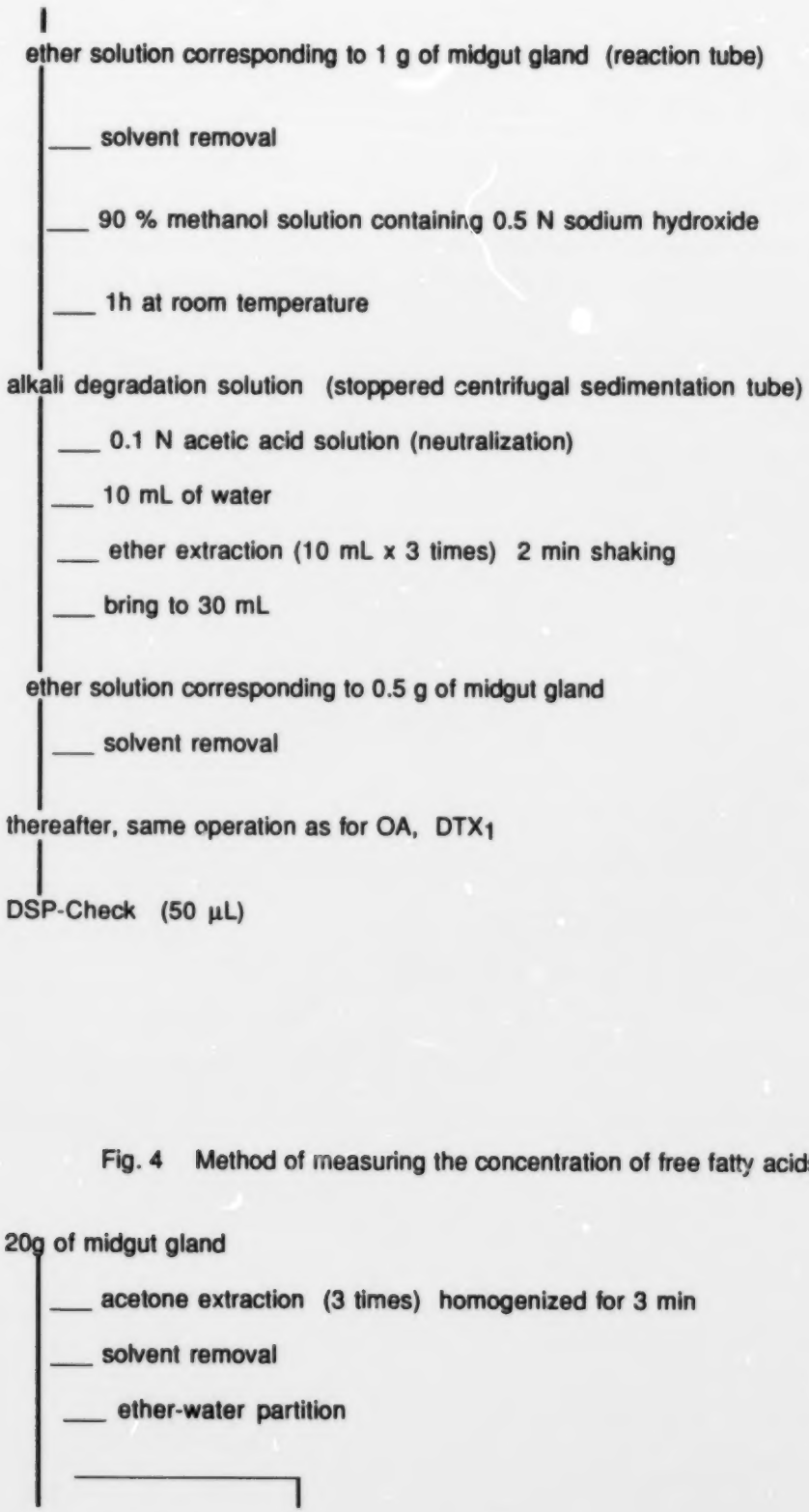

ether solution corresponding to 1 g of midgut gland (reaction tube)
___ solvent removal
___ 90 % methanol solution containing 0.5 N sodium hydroxide
___ 1h at room temperature
alkali degradation solution (stoppered centrifugal sedimentation tube)
___ 0.1 N acetic acid solution (neutralization)
___ 10 mL of water
___ ether extraction (10 mL x 3 times) 2 min shaking
___ bring to 30 mL
ether solution corresponding to 0.5 g of midgut gland
___ solvent removal
thereafter, same operation as for OA, DTX1
DSP-Check (50 µL)

Fig. 4 Method of measuring the concentration of free fatty acids

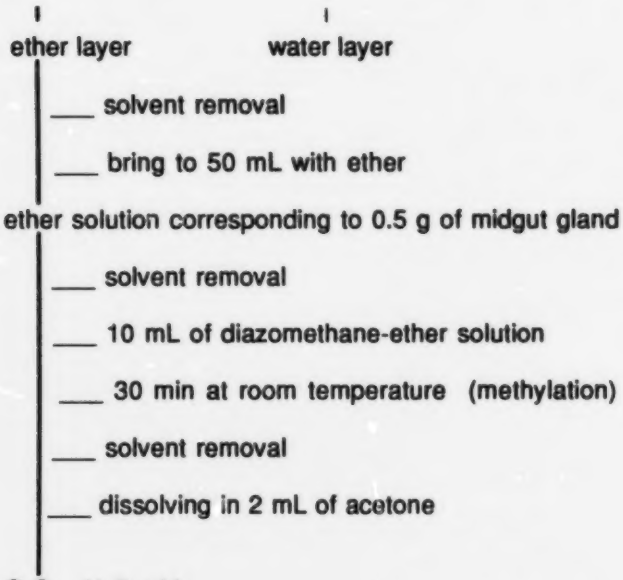

ether layer
water layer
___ solvent removal
___ bring to 50 mL with ether
ether solution corresponding to 0.5 g of midgut gland
___ solvent removal
___ 10 mL of diazomethane-ether solution
___ 30 min at room temperature (methylation)
___ solvent removal
___ dissolving in 2 mL of acetone
G-C (1-2 μL)

Fig. 5 DTX_1 virulence value at the Yanai site

_____ official method
_.—.—.— HPLC method
-------- DSP-Check method

a. midgut gland virulence
b. 1988
c. date of collection

Fig. 6 DTX_1+DTX_3 virulence values at the Nonai site

(line and letter symbols denote the same as in Fig. 5)

Fig. 7 DTX_1 virulence values at the Nobe site

(line and letter symbols denote the same as in Fig. 5)

Fig. 8 Virulence values of DTX_1 + DTX_3 at the Nobe site

(line and letter symbols denote the same as in Fig. 5)

Fig. 9 Correlation between the HPLC method and the DSP-Check method (virulence values of DTX1 + DTX 3)

a. method
b. (rate of risk significant at 1)

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Table 1 Diarrhetic shell poison virulence values at the Nonai site

a. date of collection
b. official method
c. method
d. unit
e. less than

Table 2 Diarrhetic shell poison virulence values at the Nobeji site

a. - e. : as in Table 1

Fig. 10 Free fatty acid concentrations

a. concentration
b. year 1988
c. date of collection

---------- Nonai site
_____ Nobeji site

Table 3 Results obtained by measuring the free fatty acid concentrations

a. date of collection
b. Nonai site
c. Nobeji site
d. unit

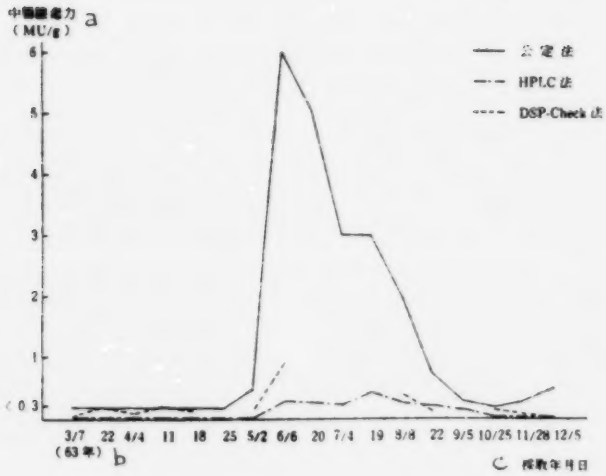

Fig. 5 図5 野内定点の DTX$_1$ 毒力値

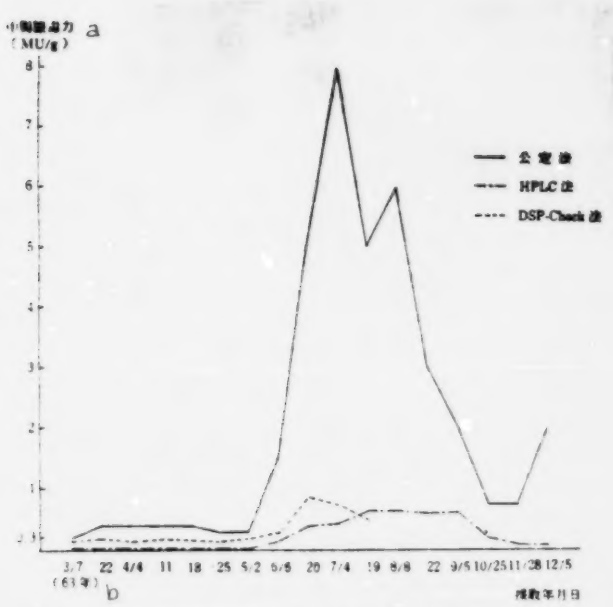

Fig. 7 図7 野辺地定点の DTX$_1$ 毒力値

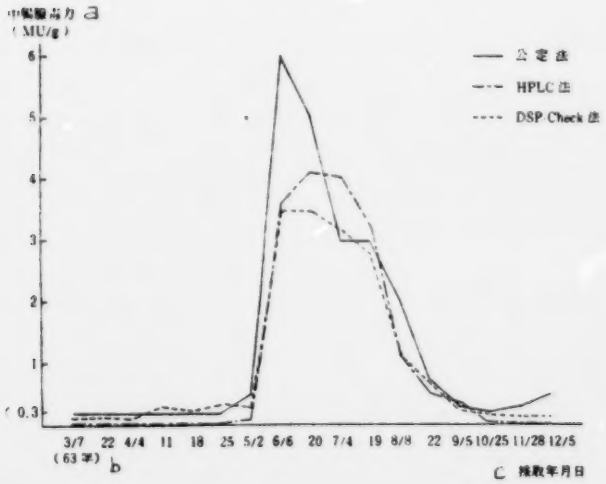

Fig. 6 図6 野内定点の DTX$_1$ + DTX$_3$ 毒力値

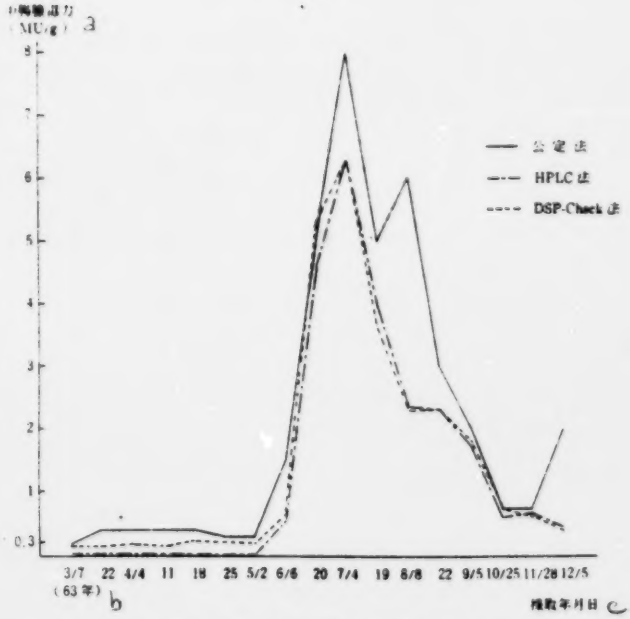

Fig. 8 図8 野辺地定点の DTX$_1$ + DTX$_3$ 毒力値

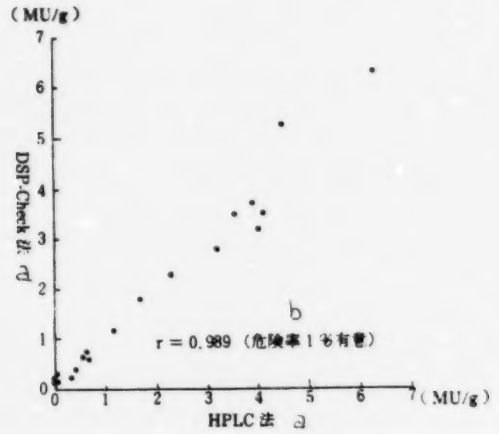

図 9　HPLC 法と DSP-Check 法の相関関係
（DTX$_1$ + DTX$_3$毒力値）b

Fig. 9

Table 1　　表 1　野内定点における下痢性貝毒毒力値

d 単位：MU/g

採取年月日 a	公定法 b	HPLC 法 c				DSP-Check 法 c			
		DTX$_1$	DTX$_1$ + DTX$_3$	DTX$_3$	$\frac{DTX_3}{DTX_1 + DTX_3} \times 100$ (%)	DTX$_1$	DTX$_1$ + DTX$_3$	DTX$_3$	$\frac{DTX_3}{DTX_1 + DTX_3} \times 100$ (%)
63. 3. 7	<0.3	ND	ND	ND	—	0.04	0.12	0.08	66.7
3.22	〃	〃	〃	〃	—	0.17	0.14	-0.03	0
4. 4	〃	〃	〃	〃	—	0.10	0.13	0.03	23.1
4.11	〃	〃	〃	〃	—	0.18	0.29	0.11	37.9
4.18	〃	〃	〃	〃	—	0.16	0.24	0.08	33.3
4.25	〃	〃	〃	〃	—	—	0.32	—	—
5. 2	0.5	〃	0.08	0.08	100	0.16	0.31	0.15	48.4
6. 6	6.0	0.28	3.60	3.32	92.2	0.9	3.5	2.6	74.3
6.20	5.0	0.27	4.13	3.86	93.5	—	3.5	—	—
7. 4	3.0	0.26	4.05	3.79	93.6	—	3.2	—	—
7.19	3.0	0.42	3.22	2.8	87.0	—	2.8	—	—
8. 8	2.0	0.28	1.18	0.9	76.3	0.4	1.2	0.8	66.7
8.22	0.75	0.19	0.56	0.37	66.1	0.14	0.65	0.51	78.5
9. 5	0.3	0.14	0.34	0.2	58.8	—	0.26	—	—
10.25	<0.3	ND	0.06	0.06	100	0.16	0.16	0	0
11.28	0.3	〃	0.02	0.02	100	0.09	0.14	0.05	35.7
12. 5	0.5	〃	0.02	0.02	100	0.03	0.16	0.13	81.2

ND：0.02 MU/g 未満　e

Table 2

表2　野辺地定点における下痢性貝毒毒力値

d 単位：MU/g

採取年月日 a	公定値 b	HPLC法 c				DSP-Check法 c			
		DTX_1	$DTX_1 + DTX_3$	DTX_3	$\frac{DTX_3}{DTX_1 + DTX_3} \times 100$ (%)	DTX_1	$DTX_1 + DTX_3$	DTX_3	$\frac{DTX_3}{DTX_1 + DTX_3} \times 100$ (%)
63. 3. 7	<0.3	ND	ND	ND	—	0.14	0.14	0	0
3.22	0.4	〃	〃	〃	—	0.20	0.14	-0.06	0
4. 4	0.4	〃	〃	〃	—	0.15	0.18	0.03	16.7
4.11	0.4	〃	〃	〃	—	0.18	0.15	-0.03	0
4.18	0.4	〃	〃	〃	—	0.18	0.23	0.05	21.7
4.25	0.3	〃	〃	〃	—	0.15	0.22	0.07	31.8
5. 2	0.3	〃	〃	〃	—	0.21	0.19	-0.02	0
6. 6	1.5	0.14	0.58	0.44	75.9	0.3	0.68	0.38	55.9
6.20	5.0	0.40	4.53	4.13	91.2	0.88	5.25	4.37	83.2
7. 4	8.0	0.44	6.31	5.87	93.0	0.75	6.3	5.55	88.1
7.19	5.0	0.62	3.94	3.32	84.3	0.52	3.7	3.18	85.9
8. 8	6.0	0.65	2.34	1.69	72.2	—	2.3	—	—
8.22	3.0	0.63	2.3	1.67	72.6	0.40	2.3	1.9	82.6
9. 5	2.0	0.62	1.72	1.1	64.0	—	1.8	—	—
10.25	0.75	0.20	0.62	0.42	67.7	0.22	0.75	0.53	70.7
11.28	0.75	0.10	0.67	0.57	85.1	—	0.62	—	—
12. 5	2.0	0.08	0.45	0.37	82.2	—	0.4	—	—

ND：0.02 MU/g 未満 e

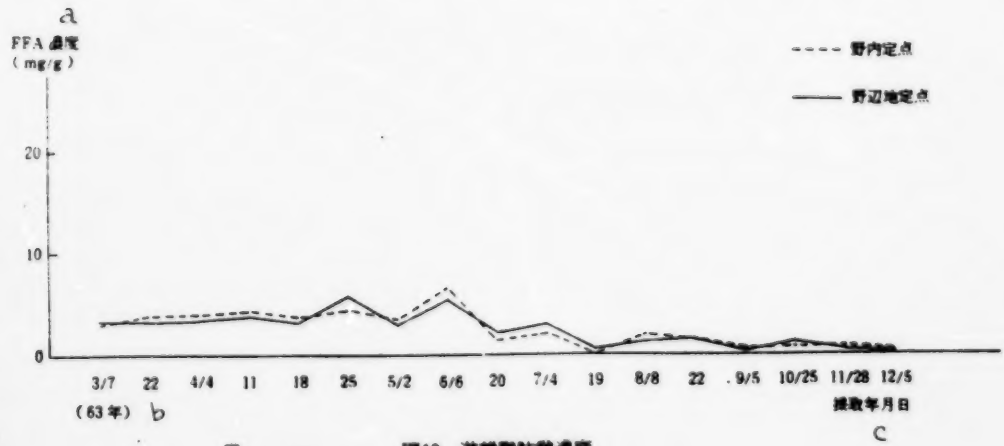

Fig. 10　図10　遊離脂肪酸濃度

Table 3　表3　遊離脂肪酸濃度の測定結果

単位：mg/g

a 採取年月日	b 野内定点	c 野辺地定点
63. 3. 7	2.94	3.17
3.22	3.85	3.34
4. 4	3.97	3.43
4.11	4.33	3.77
4.18	3.70	3.18
4.25	4.43	5.78
5. 2	3.55	2.98
6. 6	6.59	5.51
6.20	1.38	2.25
7. 4	2.14	2.96
7.19	0.11	0.58
8. 8	1.89	1.29
8.22	1.61	1.51
9. 5	0.69	0.65
10.25	0.74	1.21
11.28	0.83	0.62
12. 5	0.52	0.20

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